IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:

BAR-OR et al.

Serial No.: 10/186,168

Filed: June 27, 2002

Atty. File No.: 4172-3-3

For: "METAL-BINDING COMPOUNDS AND USES THEREFOR"

Group Art Unit: 1653

Examiner: David Lukton

DECLARATION OF DR. DAVID BAR-OR

I, David Bar-Or, M.D., declare that:

1. I am the same David Bar-Or who is named as an inventor on the above-referenced patent application. I am employed as Chief Scientific Officer and Director Of Research by DMI BioSciences, Inc. ("DMI"), the assignee of the above-referenced patent application. I am also Chairman of the Board Of Directors of DMI.

2. At my direction and under my supervision, experiments in addition to those described in the above-referenced application were performed to determine the ability of several peptides to inhibit the production of hydroxyl radicals. These additional experiments and the results of the experiments are described in Example 16 of application Serial No. 10/894,860, filed July 19, 2004, which is a continuation-in-part of this application. A true and correct copy of Example 16 is reproduced in Exhibit A, which is attached hereto and incorporated herein by reference. As can be seen from Example 16, the peptides that were tested vary by size of the peptide, sequence of the peptide (including different sizes and types of amino acids), hydrophobicity, hydrophilicity and substituents. As can also be seen from Example 16, most of the peptides gave complete inhibition

of hydroxyl radical production at peptide:copper ratios of 4:1, and several of the peptides effectively inhibited hydroxyl radical production at peptide:copper ratios of 2:1 or less.

3. The peptide L-Asp L-Ala L-His L-Lys-NH_2 (carboxyl of Lys is amidated) was tested in a rat model of myocardial ischemia and reperfusion by a third party research organization pursuant to a contract with DMI. A description of the experimental protocol is attached hereto as Exhibit B. Data received from the research organization showed that intravenous administration of the peptide caused a dose-dependent reduction in the degree of tissue necrosis caused by myocardial ischemia (25 minutes) and reperfusion (2 hours) and that all doses of the peptide (Groups 2, 3 and 4 described in Exhibit B) reduced myocardial infarct size significantly as compared to control (Group 1 described in Exhibit B). These results are also summarized in Exhibit B.

4. All statements made herein of my own knowledge are true, and all statements made on information and belief are believed to be true. I am aware that willful false statements and the like are punishable by fine, imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of this application or any patent issuing thereon.

David Bar-Or

David Bar-Or, M.D.

Date: May 31, 2005

EXHIBIT A

EXAMPLE 16: Inhibition Of The Generation Of ROS By Peptides

The ability of several peptides to inhibit the production of hydroxyl radicals was tested. The peptides that were tested are listed in Table 14 below. They were obtained from DMI Synthesis Ltd., Newport, Wales, UK. All peptides were composed of L-amino acids, unless indicated otherwise.

The ability of the peptides listed in Table 14 to inhibit the generation of hydroxyl radicals was tested. Hydroxyl radicals were generated by mixing Cu(II) and ascorbic acid. When deoxyribose was added, the hydroxyl radicals, if present, attacked the deoxyribose to produce fragments. Heating the fragments at low pH produced malonaldehyde that, upon the addition of 2-thiobarbituric acid (TBA), yielded a pink chromogen which was measured spectrophotometrically at 532 nm. Thus, absorbance at 532 nm is a measure of the damage to deoxyribose and, therefore, of hydroxyl radical formation.

To perform the assay, (a) 50 µl of 200 µM $CuCl_2$ in water, (b) water or enough of one of the test peptides in water (total volume of 150 µl) to give peptide:copper ratios of 1:4, 1:2, 1:1, 2:1, 4:1, 6:1 and 8:1, and (c) 525 µl buffer (20 mM KH_2PO_4 buffer, pH 7.4) were added to test tubes. The test tubes were incubated for 15 minutes at room temperature. Then, 25 µl of 20 mM ascorbic acid in buffer and 250 µl of 7.5 mM 2-deoxy-D-ribose in water were added to each test tube, and the test tubes were incubated for 1 hour at 37°C. Finally, 1 ml of 1% (w/v) TBA in 50 mM NaOH and 1 ml of glacial acetic acid were added to each test tube, and the test tubes were incubated in boiling water for 15 minutes. After the test tubes had cooled for 15 minutes, the absorbance at 532 nm was read. The results are presented in attached Figures 26A-V.

As can be seen from the figures, most of the peptides gave complete inhibition of hydroxyl radical production at peptide:copper ratios of 4:1. However, Ser Gly His, Thr Leu His, Ala Ala His His [SEQ ID NO:16], D-Asp Ala His Lys, Asp Ala His Lys-NH_2 [SEQ ID NO:1], Asp Ala His Gly Met Thr Cys Ala Arg Cys [SEQ ID NO:21] and Asp Ala His Gly Met Thr Cys Ala Asn Cys [SEQ ID NO:22] effectively inhibited hydroxyl radical production at peptide:copper ratios of 2:1 or less. In particular, Ala Ala His His [SEQ ID NO:16] and Asp Ala His Gly Met Thr Cys Ala Asn Cys [SEQ ID NO:22] gave complete inhibition of hydroxyl radical production at a 1:4 and 1:1 peptide:copper ratios, respectively. By comparison, the peptide Asp Ala His Lys (DAHK, both D-

and L- forms and amidated form) gave greater than 95% inhibition at a 2:1 ratio (see Figures 26 T and U and Examples 7 and 10 above).

Table 14

PEPTIDE	ABBREVIATION	FIGURE
Ala Ala His Lys [SEQ ID NO:17]	AAHK	26A
Ala Ala His Ala [SEQ ID NO:18]	AAHA	26B
Ala Ala His His [SEQ ID NO:16]	AAHH	26C
Leu Gly His	LGH	26D
Ser Gly His	SGH	26E
Asn Gly His	NGH	26F
Lys Gly His	KGH	26G
Cys Gly His	CGH	26H and 26V
Arg Thr His	RTH	26I
Ser Ser His	SSH	26J
Thr Leu His	TLH	26K
Asp Ala His Gly Gly [SEQ ID NO:19]	DAHGG	26L
Asp Ala His Gly Gly Orn Ala His [SEQ ID NO:20]	DAHGGOAH	26M
Asp Ala His Gly Met Thr Cys Ala Arg Cys [SEQ ID NO:21]	DAHGMTCARC	26N
Asp Ala His Gly Met Thr Cys Ala Asn Cys [SEQ ID NO:22]	DAHGMTCANC	26O
Asp Ala His Arg Arg Arg Arg Arg Arg [SEQ ID NO:23]	DAHRRRRRR	26P and 26V
D-Ala D-Ala D-His D-His	D-AAHH	26Q
D-Ser D-Ser D-His	D-SSH	26R
D-Phe D-Gly D-His	D-FGH	26S
D-Asp D-Ala D-His D-Lys	D-DAHK	26T
Asp Ala His Lys - NH_2 (the carboxyl of Lys is amidated) [SEQ ID NO:1]	DAHK-NH_2	26U

Figure 26A

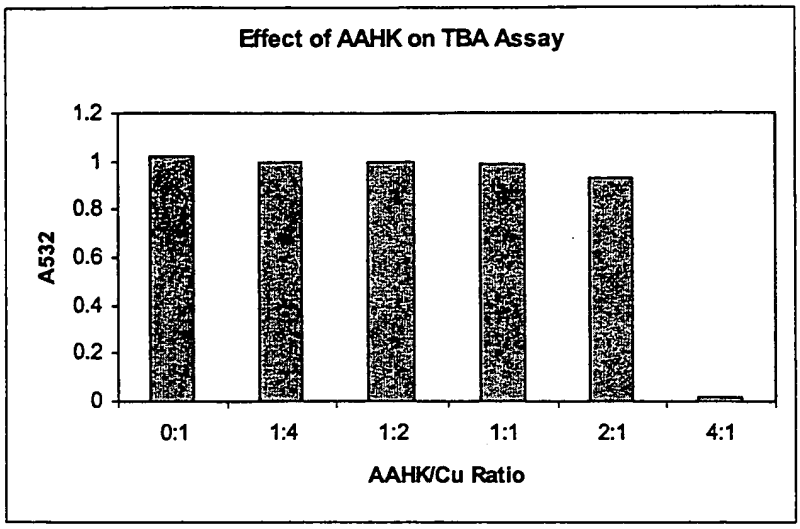


Figure 26B

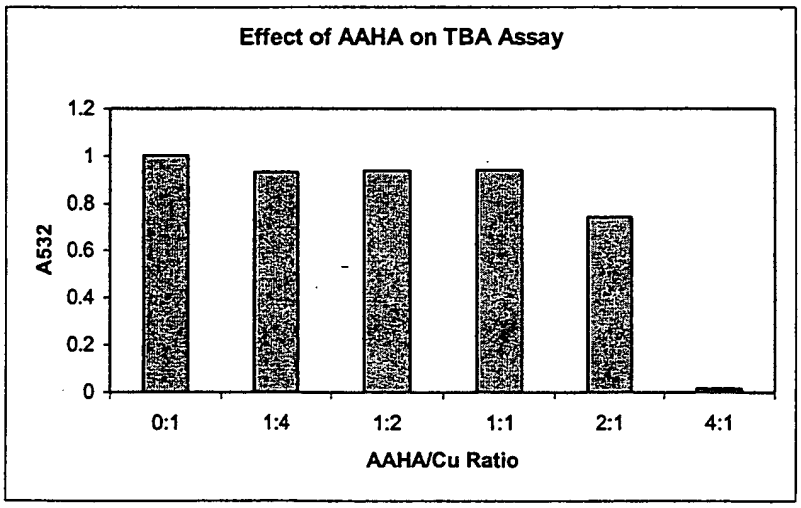


Figure 26C

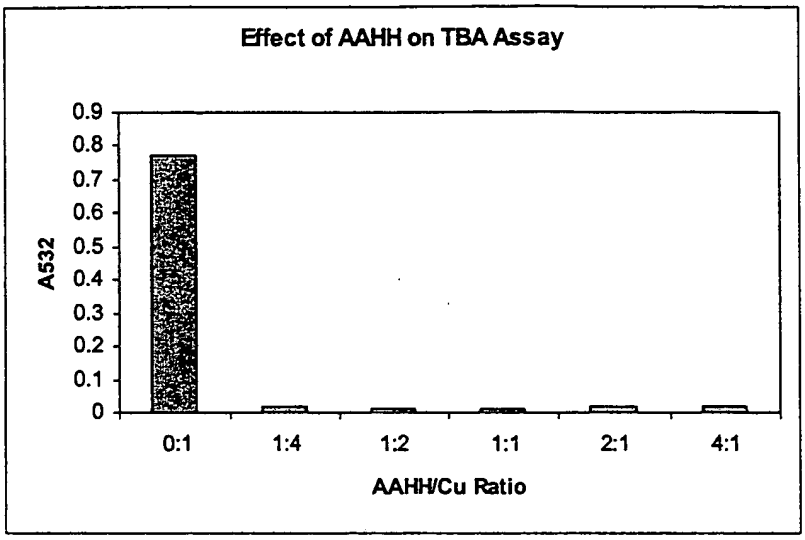


Figure 26D

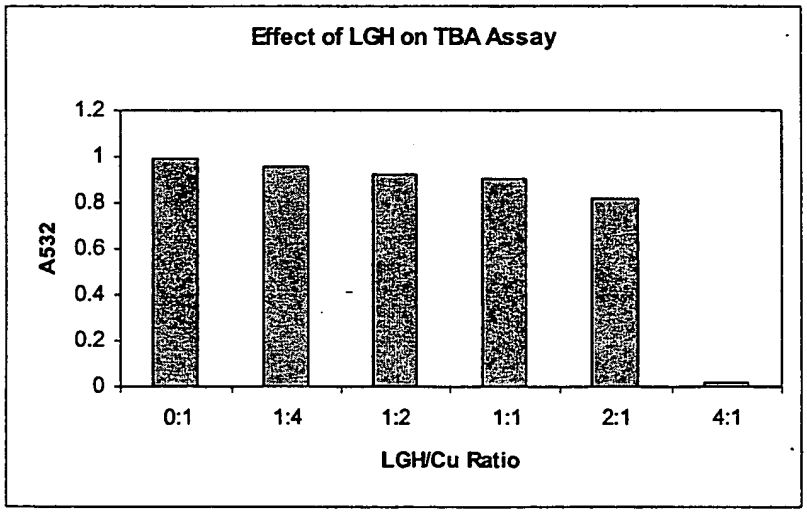


Figure 26E

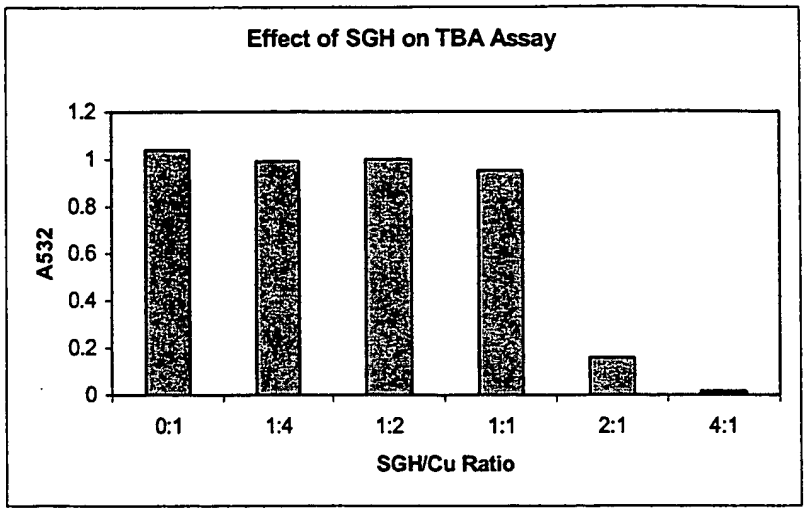


Figure 26F

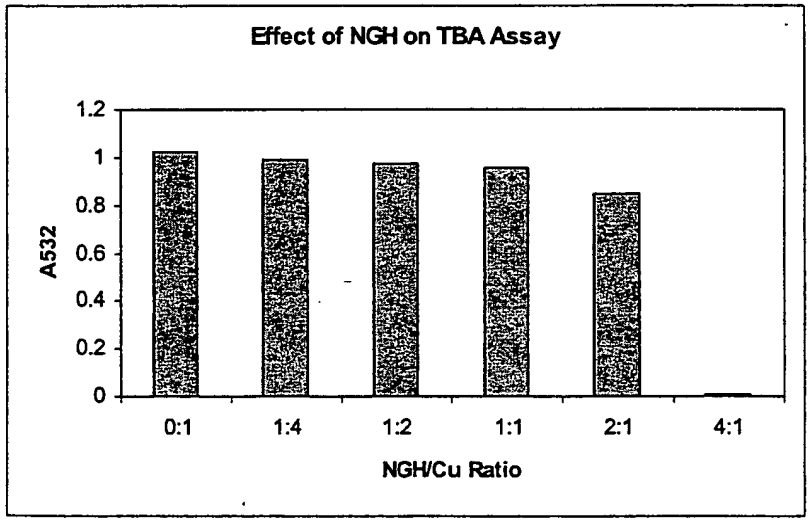


Figure 26G

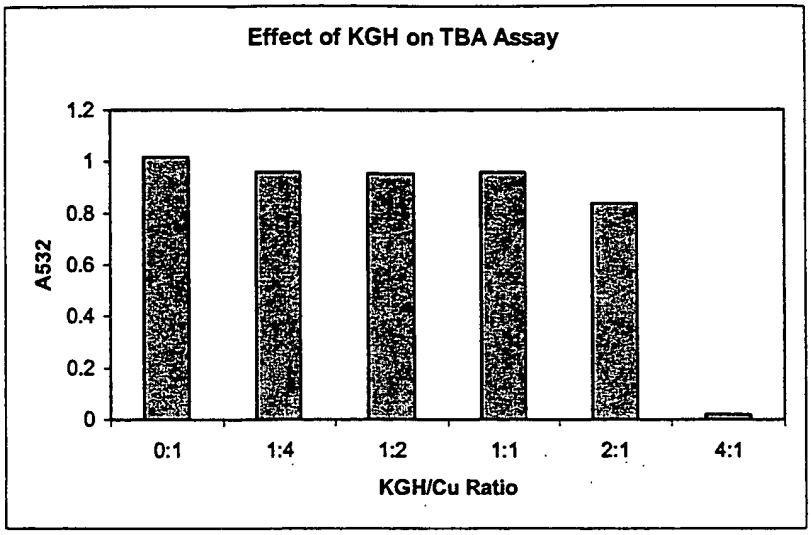


Figure 26H

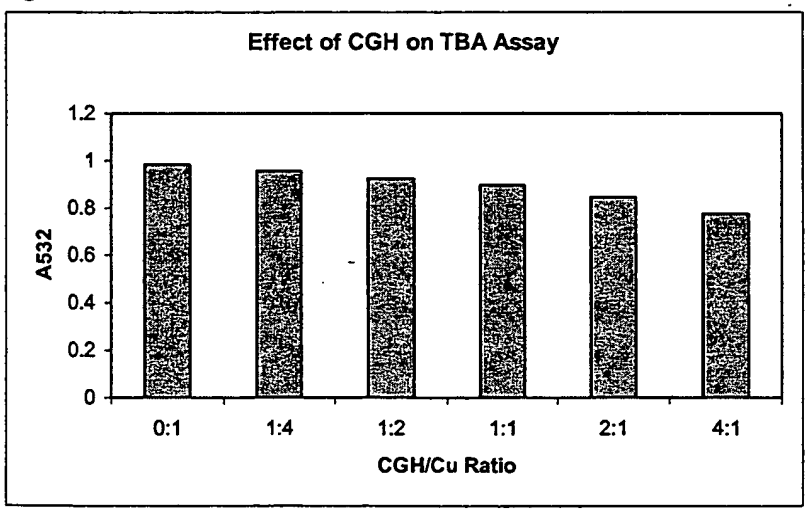
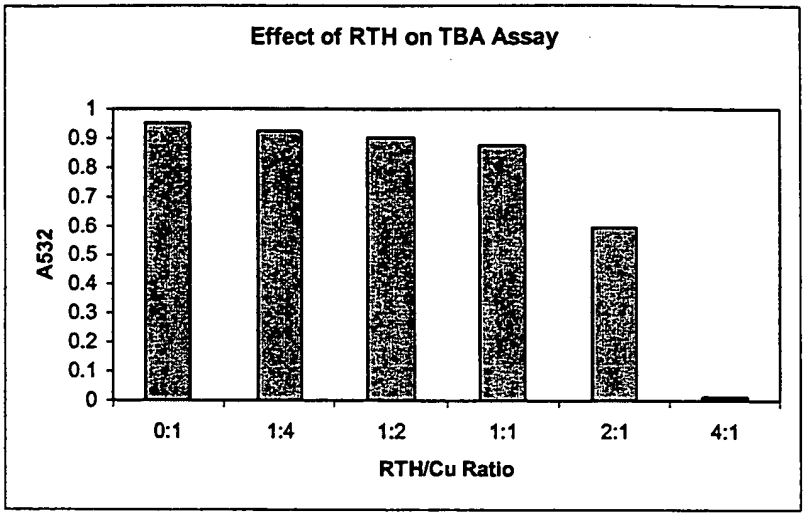


Figure 26I



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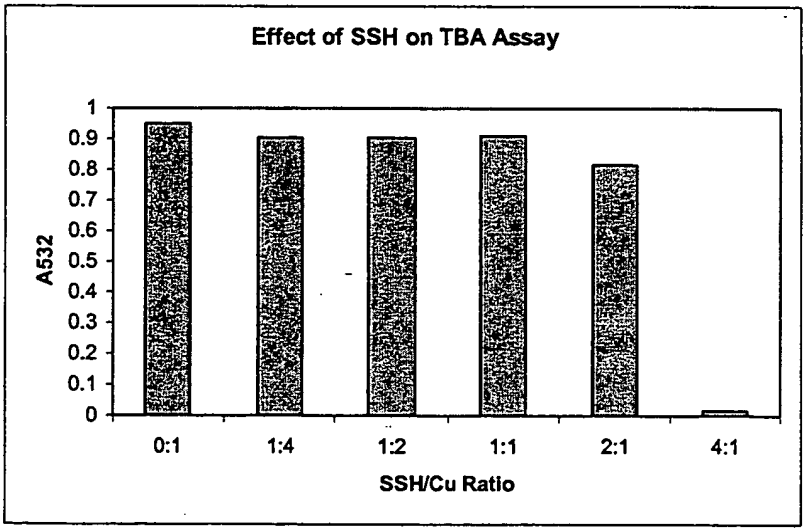


Figure 26K

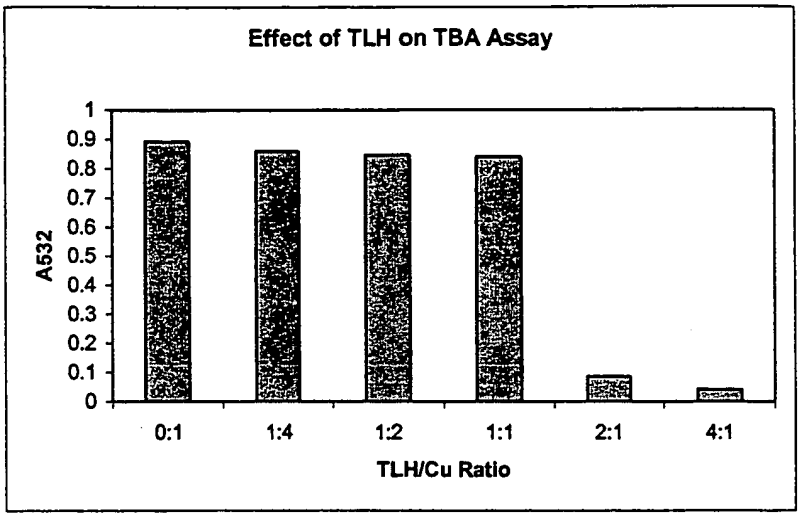


Figure 26L

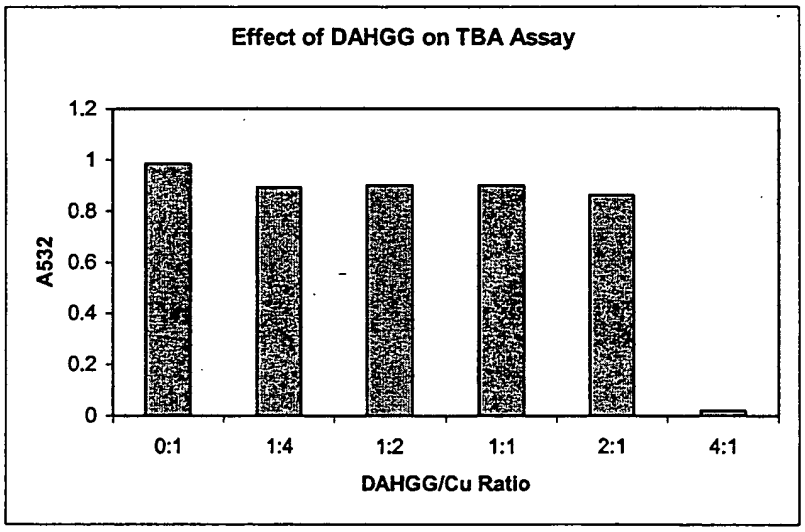
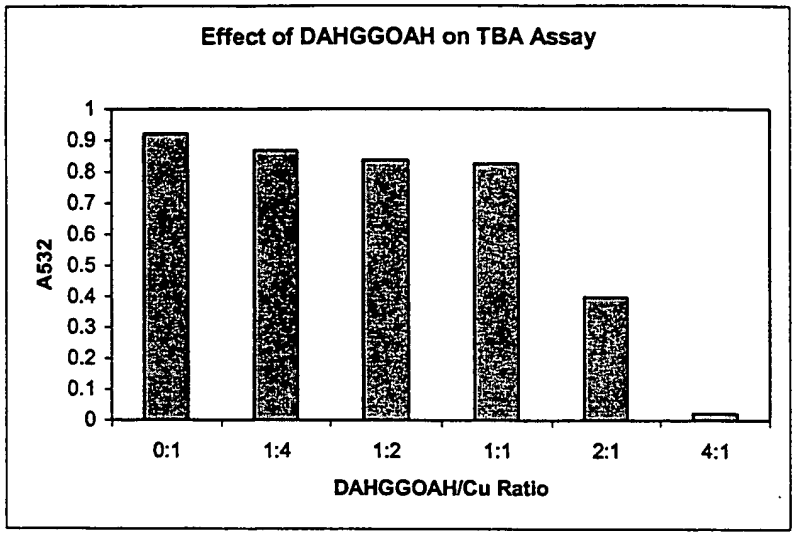


Figure 26M



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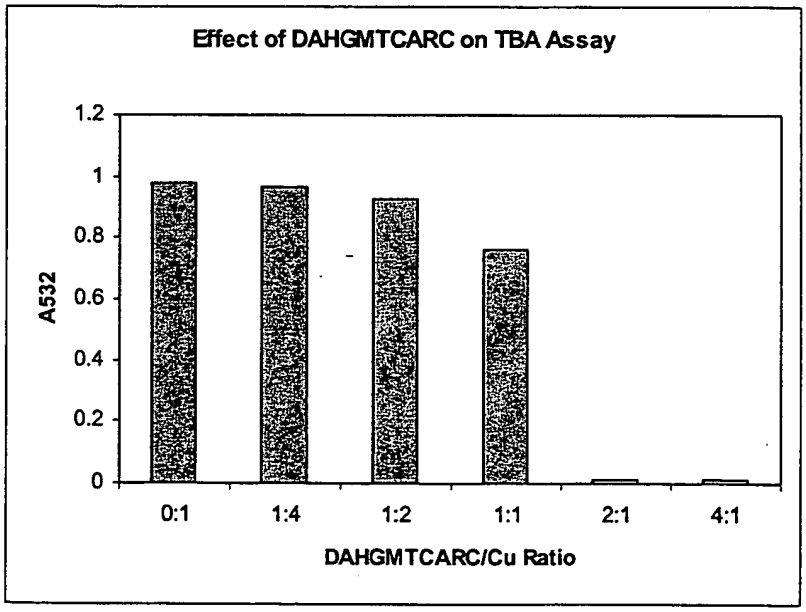

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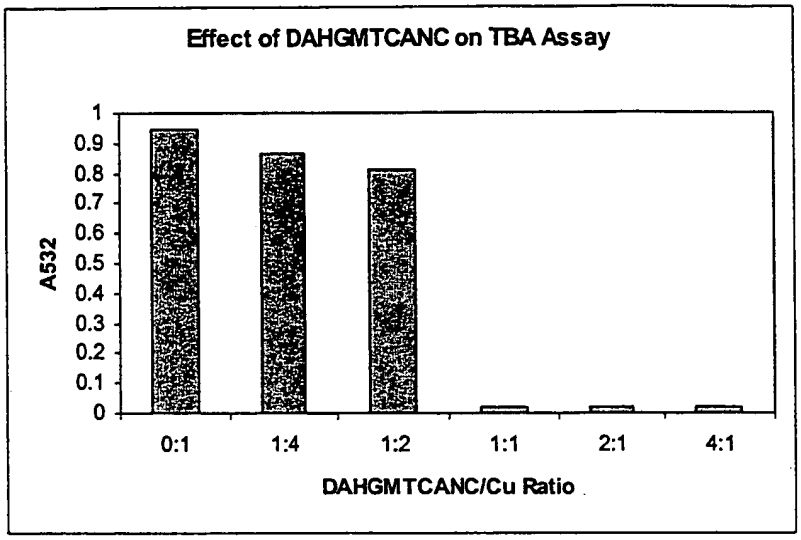

Figure 26P

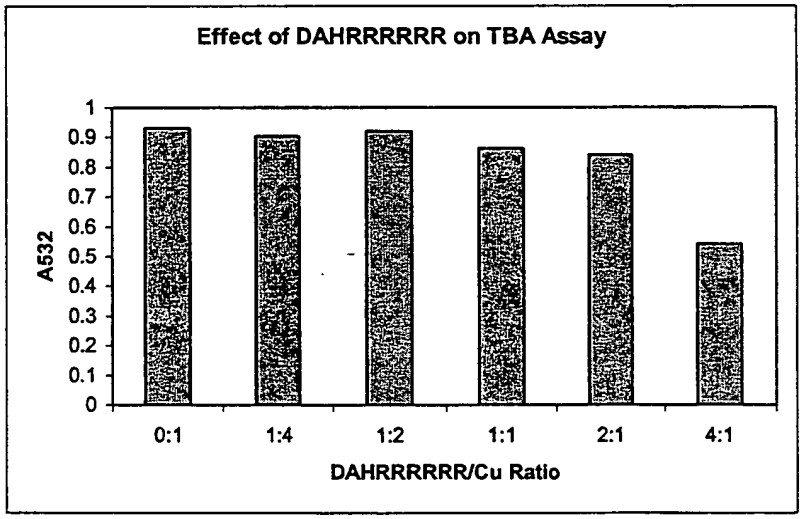

Figure 26Q

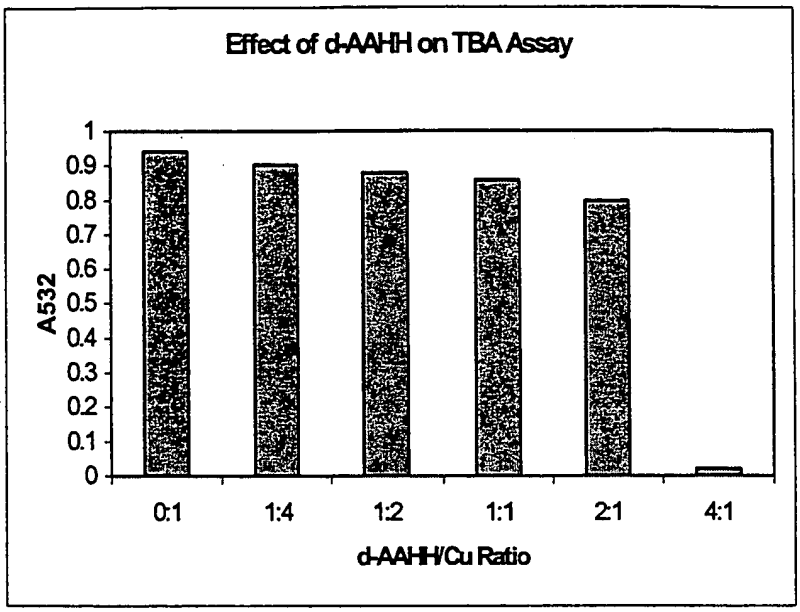

Figure 26R

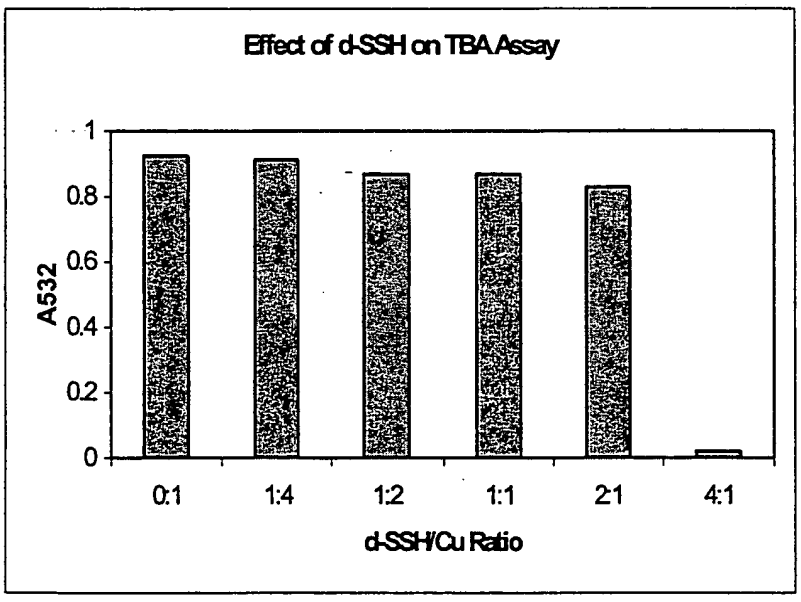

Figure 26S

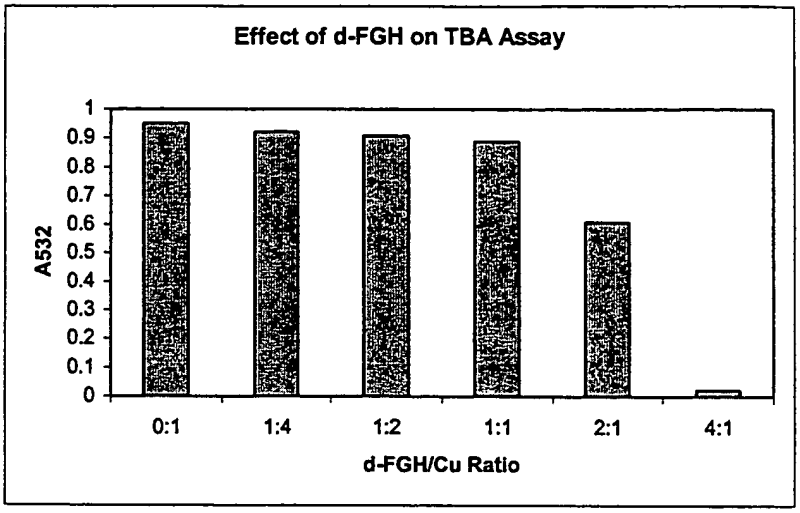

Figure 26T

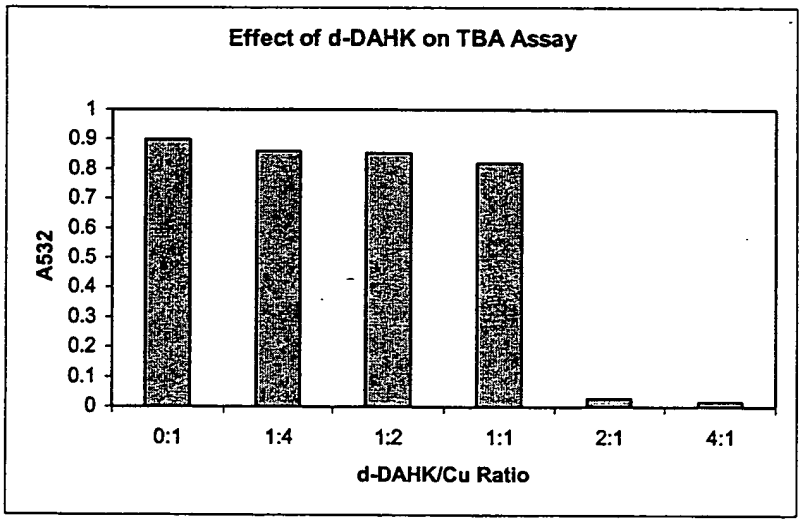

FIGURE 26U

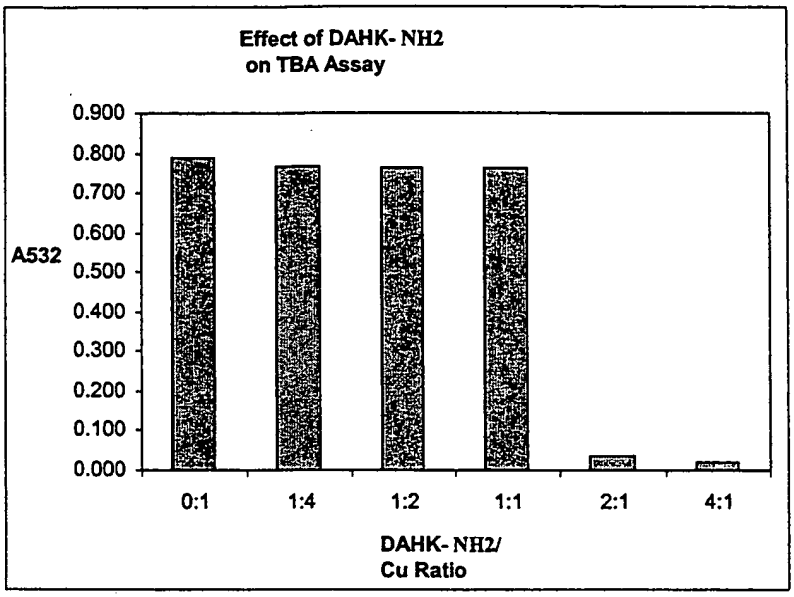

FIGURE 26V

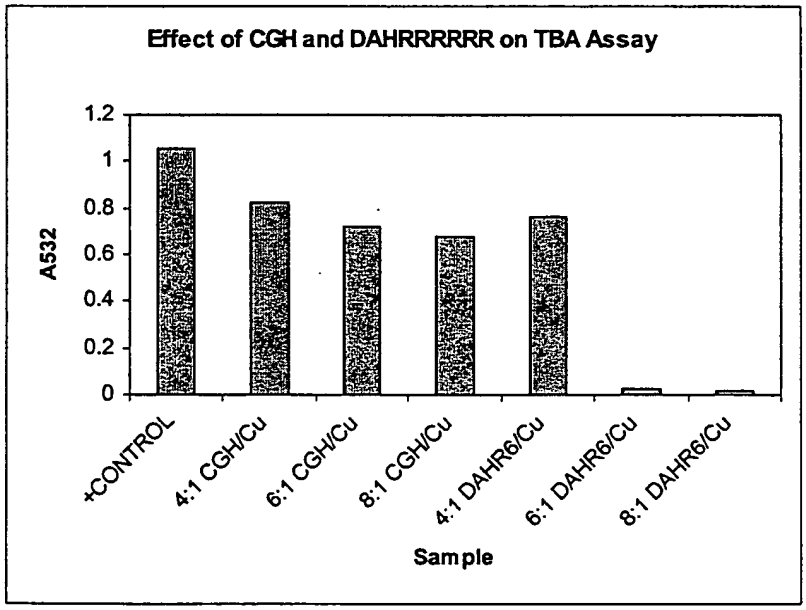

EXHIBIT B

Methods

1.1 Surgical Procedure

Thirty five male Wistar rats (200-330 g, Charles River, Margate, U.K.) were anesthetized with thiopentone sodium (Intraval®, 120 mg/kg i.p.; Rhone-Merrieux, Essex, U.K.). The rats were tracheotomized and ventilated with a Harvard ventilator (70 strokes/min, tidal volume: 8-10 ml/kg, inspiratory oxygen concentration: 30%). Body temperature was maintained at 38 ± 1°C. The right carotid artery was cannulated and connected to a pressure transducer (MLT 844, AD Instruments Ltd, Hastings, UK) to monitor mean arterial blood pressure (MAP) and heart rate (HR), which were continuously recorded on a data acquisition system (Powerlab® Version 4.0.4, AD Instruments, Hastings, UK) installed on a Dell Dimension 4100 personal computer, throughout the experiment. The right jugular vein was cannulated for the administration of test compound and Evans Blue dye (at the end of the experiment). A parasternal thoracotomy was performed, the heart was suspended in a temporary pericardial cradle and a snare occluder was placed around the left anterior descending coronary artery (LAD). After completion of the surgical procedure the animals were allowed to stabilize for 20 min before LAD ligation. The coronary artery was occluded at time 0 by tightening of the occluder. After 25 minutes of acute myocardial ischemia, the occluder was re-opened to allow reperfusion for 2 hours.

The test compound, L-Asp L-Ala L-His L-Lys-NH_2, was administered as a continuous intravenous (i.v.) infusion of 10 mg/kg/h starting at 19 minutes after the onset of myocardial ischemia, which continued throughout the remainder of myocardial ischemia and reperfusion. A slow (i.v.) bolus injection was administered at 20 minutes after the onset of myocardial ischemia. Following the 2 hour reperfusion period, the coronary artery was re-occluded and Evans Blue dye (1 ml of 2% w/v) was injected into the left ventricle, via the right jugular vein cannula, to distinguish between perfused and non-perfused (AAR) sections of the heart. The Evans Blue solution stains the perfused myocardium, while the occluded vascular bed remains uncolored. The animals were killed with an overdose of anaesthetic and the heart excised. The heart was sectioned into

slices of 3-4 mm, the right ventricular wall was removed, and the AAR (pink) was separated from the non-ischemic (blue) area. The AAR was cut into small pieces and incubated with *p*-nitroblue tetrazolium (NBT, 0.5 mg/ml) for 40 minutes at 37°C. In the presence of intact dehydrogenase enzyme systems (viable myocardium), NBT forms a dark blue formazan, whilst areas of necrosis lack dehydrogenase activity and therefore fail to stain. Pieces were separated according to staining and weighed to determine the infarct size as a percentage of the weight of the AAR.

1.2 Experimental Design

To elucidate the effects of the synthetic peptide, L-Asp L-Ala L-His L-Lys-NH_2, on the infarct size caused by regional myocardial ischemia and reperfusion, all animals were randomized into 4 groups (see Table 1, where N denotes the number of animals used in the study, and n represents the number of survivors).

The first group comprised of animals subjected to regional myocardial ischemia (25 minutes) followed by reperfusion (2 hours) and treated with vehicle (saline, 1 ml/kg followed by an infusion of 1 ml/kg/h i.v. throughout the remainder of ischemia and all of reperfusion).

The second group of animals was subjected to regional myocardial ischemia (25 minutes) followed by reperfusion (2 hours) and was treated with L-Asp L-Ala L-His L-Lys-NH_2 (**5 mg/kg**, 20 minutes after the onset of myocardial ischemia, followed by an infusion of 10 mg/kg/h throughout the remainder of ischemia and all of reperfusion).

The third group of animals was subjected to regional myocardial ischemia (25 minutes) followed by reperfusion (2 hours) and was treated with L-Asp L-Ala L-His L-Lys-NH_2 (**10 mg/kg**, 20 minutes after the onset of myocardial ischemia, followed by an infusion of 10 mg/kg/h throughout the remainder of ischemia and all of reperfusion).

Similarly, the fourth group of animals was subjected to regional myocardial ischemia (25 minutes) followed by reperfusion (2 hours) and was treated with L-Asp L-Ala L-His L-Lys-NH_2 (**20 mg/kg**, 20 minutes after the onset of myocardial ischemia, followed by an infusion of 10 mg/kg/h throughout the remainder of ischemia and all of reperfusion).

See Table 1 for a summary of the experimental groups.

Table 1

Group	Group I.D.	Treatment	Dose	N	n
1	Vehicle	Vehicle (0.9% NaCl pH 7.4)	1 ml/kg + 1 ml/kg/h	9	8
2	L-Asp L-Ala L-His L-Lys-NH_2 5 mg/kg + 10 mg/kg/h	L-Asp L-Ala L-His L-Lys-NH_2	5 mg/kg + 10 mg/kg/h	9	9
3	L-Asp L-Ala L-His L-Lys-NH_2 10 mg/kg + 10 mg/kg/h	L-Asp L-Ala L-His L-Lys-NH_2	10 mg/kg + 10 mg/kg/h	8	8
4	L-Asp L-Ala L-His L-Lys-NH_2 20 mg/kg + 10 mg/kg/h	L-Asp L-Ala L-His L-Lys-NH_2	20 mg/kg+ 10 mg/kg/h	9	9

1.3 Statistical evaluation

All data are presented as mean ± s.e.mean of *n* observations, where *n* represents the number of animals studied. Infarct size was analyzed by 1-factorial analysis of variance (ANOVA), followed by a Dunnett's test for comparison of a treated group to the vehicle. Hemodynamic data was analyzed by 2-way ANOVA followed by a Bonferroni's test. A *P*-value of less than 0.05 was considered to be statistically significant.

Results: *Effects of the test compound, L-Asp L-Ala L-His L-Lys-NH_2, on the infarct size caused by regional myocardial ischemia and reperfusion*

The area at risk of infarction (AAR) was similar in all groups studied and ranged from 52 ± 2 to 54 ± 2% of the left ventricle ($P>0.05$). In rats, which were treated with vehicle, occlusion of the LAD (for 25 minutes) followed by reperfusion (for 2 hours) resulted in an infarct size of **64 ± 3%** of the AAR (n=8). Intravenous administration of the lowest dose of L-Asp L-Ala L-His L-Lys-NH_2 (5 mg/kg followed by 10 mg/kg/h) reduced the infarct size from 64 ± 3 to **52 ± 3** of the AAR ($P<0.05$ when compared to vehicle). Intravenous administration of the median dose of L-Asp L-Ala L-His L-Lys-NH_2 (10 mg/kg followed by 10 mg/kg/h) reduced the infarct size to **50 ± 2** of the AAR ($P<0.05$ when compared to vehicle). Intravenous administration of the highest dose of L-Asp L-Ala L-His L-Lys-NH_2 (20 mg/kg followed by 10 mg/kg/h) reduced the infarct size to **45 ± 3** of the AAR ($P<0.05$).

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